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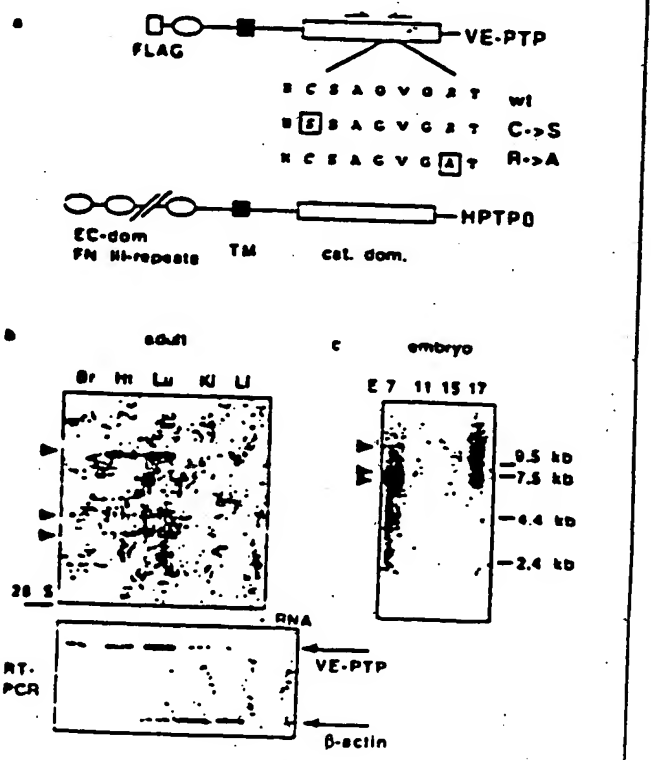
(54) Title: INTERACTION OF VASCULAR-ENDOTHELIAL PROTEIN-TYROSINE PHOSPHATASE WITH THE ANGIOPOIETIN RECEPTOR TIE-2

(57) Abstract

Use of vertebrate vascular-endothelial protein tyrosine phosphatases (i.e. murine phosphatase VE-PTP or human phosphatase HPTPβ) or portions thereof for the manufacture of an agent for monitoring or modulating the activity of the angiopoietin receptor-type tyrosine kinase Tie-2.



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Interaction of vascular-endothelial protein-tyrosine phosphatase with the Angiopoietin receptor Tie-2

Specification

The present invention relates to a method for monitoring or modulating the activity of the angiopoietin receptor-type tyrosine kinase Tie-2.

A key mechanism in the proliferation and differentiation control of all cells are membrane-located receptors, whose activation in many cases is mediated by external factors via phosphorylation of tyrosine residues. The mutation of a series of endothelial cell specific receptor-tyrosine kinases (RTKs) results in lethal phenotypes early during murine embryonal development (Hanahan, Science 277 (1997), 48 - 50; Risau, Nature 386 (1997), 671 - 674). The proliferation and differentiation of endothelial cells depends on two receptor tyrosine kinase systems. The vascular endothelial growth factor (VEGF) is a secreted angiogenic factor and promotes vascularization by activation of its high affinity receptors VEGFR-1 (Flt-1) or VEGFR-2 (Flk-1). The RTKs Tie-1 and Tie-2 are involved in the sprouting and remodelling of the embryonic vascular system. The activity of these kinases is regulated by the recently identified ligands, the angiopoietins.

After ligand binding RTKs are activated by phosphorylation on tyrosine residues. Specific protein-tyrosine phosphatases (PTPs) are involved in the fine-tuning of RTK activity. Several classes of PTPs have been identified. However, the biological functions thereof are presently not understood (Neel & Tonks, Curr. Opin. Cell Biol. 9 (1997), 193 - 204; Streuli, Curr. Opin. Cell Biol. 8 (1996), 182 - 188).

In a study to identify PTPs in endothelial cells a murine vascular-endothelial protein-tyrosine phosphatase VE-PTP was identified (VE-PTP: a receptor

protein-tyrosine phosphatase expressed in vascular endothelium, EMBO-FEBS Workshop on Protein Phosphatases and Protein Dephosphorylation, Oxford, UK, September 21 - 26, 1997). Indications for a functional interaction between VE-PTP and a receptor-type kinase have not been described, however. Further, the association of PTPs with their substrates is difficult to determine due to the transcient nature of the enzyme substrate association (Flint et al., Proc. Natl. Acad. Sci. U.S.A. 94 (1997), 1680 - 1685).

The experiments underlying the present application discovered that VE-PTP is a homolog of the human HPTPβ (Krueger et al., EMBO J., 9, (1990), 3241 - 3252), and that it is specifically expressed in endothelial cells both during the embryonal development of mice and in brain capillary vessels of newborn animals. Biochemical analyses using VE-PTP trapping mutants show a specific interaction between the C-terminal part of the molecule which contains the catalytic domain and the RTK Tie-2 but not with the vascular endothelial growth factor receptor VEGFR-2. Moreover, a dephosphorylation of Tie-2 could be detected in the presence of VE-PTP in transiently transfected COS-1 cells. These data identify Tie-2 as a specific substrate for VE-PTP and show that it is a specific modulator of Tie-2 activity.

This result is of high clinical relevance, as Tie-2 holds a key position in angiogenetic processes, the formation of the blood vessel system during embryonal development, the healing of wounds as well as in pathological processes, e.g. tumor development. As VE-PTP shows a specific interaction with Tie-2 and can modulate the tyrosine phosphorylation of the latter, the receptor-protein tyrosine phosphatase is a target both for diagnostic monitoring and for therapeutically influencing the said processes.

Thus, a subject matter of the present invention is the use of vertebrate, e.g. mammalian vascular-endothelial protein-tyrosine phosphatases or portions

thereof for the manufactur of an agent for monitoring or modulating the activity of receptor-type tyrosine kinase Tie-2.

A further subject matter of the present invention is the use of nucleic acids encoding vertebrate, e.g. mammalian vascular-endothelial protein-tyrosine phosphatases or portions thereof for the manufacture of an agent for monitoring or modulating the activity of receptor-type tyrosine kinase Tie-2.

Still a further subject matter of the invention is the use of ligands for vertebrate, e.g. mammalian vascular-endothelial protein-tyrosine phosphatases for the manufacture of an agent for monitoring or modulating the activity of receptor-type tyrosine kinase Tie-2.

The vascular-endothelial protein-tyrosine phosphatases and nucleic acids coding therefor, e.g. genes or cDNA molecules, are obtainable from vertebrate cells, preferably from mammalian endothelial cells, e.g. murine or human cells. Preferably the vascular-endothelial protein-tyrosine phosphatase is selected from murine phosphatase VE-PTP, human phosphatase HPTPβ or portions thereof, particularly portions comprising the catalytic domain which is located at the C-terminus of the molecule (Fig. 1a). The nucleic acid sequence and the corresponding amino acid sequence of murine vascular-endothelial protein-tyrosine phosphatase are depicted in SEQ. ID. NO 1 and 2, respectively. The corresponding sequences of the human protein, which were identified by Krueger et al. (supra) are depicted in SEQ. ID. NO 3 and 4.

The polypeptide or a portion thereof is suitable for monitoring or modulating the activity of receptor-type tyrosine kinase Tie-2. In addition to a phosphatase with unmodified sequence of the catalytic domain also mutants thereof, which show a modified, e.g. enhanced binding to Tie-2, e.g. the trapping mutants as depicted in Fig. 2 are suitable for the present

invention. Particularly mutants, which exhibit an enhanced binding to Tie-2 are well suited for diagnostic and therapeutic applications.

The interaction between the vascular endothelial protein-tyrosine phosphatase and its substrate Tie-2 can also be monitored and/or modulated on the nucleic acid level. To this end nucleic acids, e.g. DNA molecules, RNA molecules or artificial nucleic acid analogs such as peptidic nucleic acids may be used. Preferably these nucleic acids comprise at least 15, particularly at least 20 nucleotides from murine phosphatase VE-PTP gene, human phosphatase HPTPβ gene or sequences complementary thereto. These nucleic acids are suitable for the determination of the PTP expression by using known hybridization or/and amplification techniques such as PCR. On the other hand, nucleic acids can be used for the modulation of the VE-PTP expression in the form of antisense constructs or as ribozymes.

A still further aspect of the invention is the use of ligands for vertebrate, e.g. mammalian vascular endothelial-protein tyrosine phosphatases. Examples of such ligands are antibodies, e.g. polyclonal or monoclonal antibodies and antibody fragments. Polyclonal antibodies are available according to known protocols by immunization of test animals with purified VE-PTP, HPTPβ or partial fragments thereof, which preferably contain the catalytic domain. From these test animals monoclonal antibodies can be generated in a known manner by using the method applied by Koehler and Milstein. The polyclonal or monoclonal antibodies can also be used in the form of fragments which are obtainable by proteolyic treatment or genetic engineering.

One embodiment of the invention concerns the monitoring or detection of the Tie-2 activity. This detection can be carried out by using kn wn methods, e.g. using labelled polypeptides, nucleic acids or antibodies. A

further embodiment concerns the modulation of the Tie-2 activity. Thereby a stimulation or a repression of the Tie-2 activity is possible.

Of major importance is the examination or influencing of the interaction between VE-PTP and Tie-2 for angiogenesis. Thus the present invention provides means for inducing or for inhibiting vascular growth or remodelling and blood vessel maturation. Particularly, the present invention provides means for inhibiting tumor growth and formation of tumor metastases, e.g. by repressing Tie-2 activity in target cells.

Moreover, the invention is explained by the following figures and sequence protocols.

Fig. 1a shows the schematic representation of VE-PTP, its genetically engineered trapping mutants and HPTPβ.

Fig. 1b and c show Northern blot and RT-PCR analyses of VE-PTP expression in mouse tissues and during mouse embryonic development.

Fig. 2 shows *in vivo* expression analysis of VE-PTP by *in situ* hybridization.

Fig. 3 shows biochemical interactions of VE-PTP trapping mutants with Tie-2 protein.

Fig. 4 shows selective dephosphorylation of Tie-2, but not VEGFR-2 by wild-type VE-PTP.

Fig. 5 shows a sequence comparison of the C-terminus of HPTPβ with VE-PTP and the translated "mRPTPβ" sequence. Known protein domains are depicted:

Membrane proximal FN III-domain (blue), transmembrane domain (red) and catalytic domain (green). The catalytic center is characterized by a $C(x)_5R$-motif.

SEQ. ID. NO. 1 and 2 show the nucleotide sequence of VE-PTP cDNA and the corresponding amino acid sequence.

SEQ. ID. NO. 3 and 4 show the nucleotide sequence of HPTPβ cDNA and the corresponding amino acid sequence.

Example 1

A PCR screen of a murine brain capillary cDNA library and reverse transcribed mRNA of bEND5 endothelioma cells to identify endothelial specific members of the protein-tyrosine phosphatase family was performed. For PCR, 100 pmol degenerated primers RPTP1 5'-GA(C/T) TT(C/T) TGG ATG (A/G/T) (G/T)I TGG GA-3' and RPTP2 5'-CCI ACI CGI GCI (G/C)(A/T)(A/G) CA(A/G) TGI AC-3' in 50 µl reactions were used. As templates 1.25 µg λ-DNA from mouse P4-10 brain capillary-library (Schnürch & Risau, Development, 119 (1993), 957 - 968) or 3 µl of SuperScript cDNA preparation (GIBCO BRL) from bEND5 mRNA were used. Isolated 370 bp products were cloned into the vector pCRII (Invitrogen), analysed by restriction cleavage and sequenced on an ABI 370 automated sequencer (Applied Biosystems).

One of the identified PCR products encodes a polypeptide, designated as vascular-endothelial protein-tyrosine phosphatase (VE-PTP) which was identified as murine homolog of the previously described receptor-type protein-tyrosine phosphatase HPTPβ (Krueger et al. EMBO J. 9 (1990), 3241 - 3252). VE-PTP and HPTPβ belong to the subclass III of receptor-type PTPs bearing exclusively fibronectin type III-like repeats in the extracellular

domain and a single catalytic domain in the cytoplasmatic tail (Fig. 1a) (Brady-Kalnay & Tonks, Curr. Opin. Cell. Biol. 7 (1995), 650 - 657).

Fig. 1a shows a schematic representation of VE-PTP, its genetically engineered trapping mutants C->S, R->A and HPTPβ. Rectangles indicate mutated amino acids in the catalytic core. The location of the degenerated primers used in the PCR screen are indicated by arrows (EC-dom., extracellular domain; FN III fibronectin-type III-like repeat; cat. dom., catalytic domain).

Example 2

A Northern blot and RT-PCR analysis of VE-PTP expression in mouse tissues and during mouse embryonic development were performed. A 751 bp EcoRI-fragment from VE-PTP part 1, obtained by PCR using primers PrPTP$\beta$for 5'-GGA AGA GGT ACC TGG TGT CCA TCA AGG-3' and PrPTPβrev 5'-GGC CGG TCC CTA CGA ATG CTG AGC CGG GCA G-3' deduced from a partial clone of murine "RPTPβ" (Schepens et al. Mol. Biol. Reports, 16 (1992)), and cloned in the vector pBS KS(+)(Stratagene), was labelled with α^{32}P-dCTP (Amersham Pharmacia Biotech). For Northern blot analysis 20 μg of total RNA from mouse tissues (Chomczynski & Sacchi, Analyt. Biochem. 162 (1987), 156 - 159) were loaded on a formaldehyde containing agarose gel and blotted. A mouse embryo mRNA Northern blot was obtained from Clontech and hybridization was carried out according to manufacturer's instructions. Autoradiography was performed at -70° C for 17 d. For semiquantitative PCR 50 μl reactions containing 2 μl of reverse transcribed cDNA preparations and 20 pmol of primers betaseq2 5'- CCC TCT CCC TTC CTA CCT GG-3' and betarev 5'- GGC CGG TCC CTA CGA ATG CTG AGC CGG GCA GG-3' were used, giving a 416 bp fragment. 30 cycles PCR was optimized to detect 1 fg of VE-PTP plasmid DNA. β-actin RT-PCR was performed as described (Nakajima-Iijima et al, Proc. Natl. Acad. Sci. U.S.A. 82 (1985), 6133 - 6137).

Northern blot analysis of VE-PTP expression revealed a major transcript of approximately 11 kB and two additional transcripts of 7.5 and 6 kB. In the adult mouse VE-PTP mRNA was strongly expressed in brain as well as in lung and heart. Very weak expression was detectable in kidney and liver (Fig. 1b). These data were confirmed by semi-quantitative RT-PCR performed with RNA from these organs (Fig. 1b). During embryonic development VE-PTP was weakly expressed at embryonic day E11, expression increased at E15 reaching a maximum at E17 (Fig. 1c). Strong expression was detected at E7, which may result from expression in contaminating maternal tissue as expression in the placenta was observed by *in situ* hybridization analysis as well.

Example 3

An *in vivo* expression analysis of VE-PTP by *in situ* hybridization to frozen sections of mouse embryonic tissues was carried out. The results are shown in Fig. 2. Fig. 2a is a darkfield image of an E12.5 embryo section hybridized with a VE-PTP antisense probe. (NC: neural crest, DA: dorsal aorta). Fig. 2b is a darkfield image and Fig. 2c is a brightfield image of a higher magnification of the vessel indicated in a (asteriks). Fig. 2d - h are sagittal sections of E15.5 embryos hybridized with antisense VE-PTP probes. Fig. 2d is a darkfield image and Fig. 2e a brightfield image of the lung. Fig. 2f is a darkfield image of the head region. Fig. 2g is an E15.5 embryo section hybridized with a VEGFR-2 antisense probe. Fig. 2h - k are vessels in brain sections of P10 mice hybridized with antisense VE-PTP probes. As templates for *in vitro* transcription pCRII (Invitrogen) VE-PTP-1 (370 bp fragment of VE-PTP coding for protein sequence corresponding to aa 1786 - 1913 in HPTPβ in pCRII) and pBS VE-PTPpart1 were used. Sectioning of mouse embryos and *in situ* hybridization were performed as described (Breier et al, Development, 114 (1992), 521 - 532).

At the earliest timepoint analysed (E9.5), expression was detectable in the endothelial cell layer lining the dorsal aortae. During the subsequent developmental stages VE-PTP expression was increased throughout the developing vascular system (Fig. 2a). Strong hybridization signals were visible in endothelial cells forming blood vessels, whereas no specific signals were detected in blood cells or smooth muscle cells surrounding the vessels (Fig. 2b, c). At E15.5 specific signals were detectable in all organs with highest expression in the lung (Fig. 2d,e). Comparison to serial sections hybridized with an antisense probe to VEGFR-2 (Flk-1) as an endothelial cell marker, confirmed the vascular endothelial specific expression pattern of VE-PTP (Fig. 2 f,g). In contrast to the uniform expression levels of VEGFR-2 in different types of embryonic endothelial cells, VE-PTP was more strongly expressed in endothelial cells lining larger, smooth muscle cell invested vessels than those of small capillaries and veins. On brain sections of newborn mice, specific expression of VE-PTP was detectable in brain capillaries as well as in larger vessels (Fig. 2h-k). No specific signals were visible in neuronal or glial cells.

Example 4

The biochemical interactions of VE-PTP with the receptor tyrosine kinases Tie-2 and VEGFR-2 were investigated using bacterial GST-fusion proteins. The results are shown in Fig. 3.

Fig. 3a demonstrates the results of GST-fusion pull down experiments. GST and GST x VE-PTP R/A fusion protein were incubated with lysates from bEND5 cells. Precipitates were blotted with an anti-Tie-2 antibody and reblotted with an VEGFR-2 specific antibody. (tot. lys.: total lysates of bEND5 cells). pGEX-VE-PTP contains a 1.1 kB 3' part of EST-clone 552065 (Lennon et al., Genomics 33 (1996), 151 -152) coding for the cytoplasmic domain of VE-PTP cloned in pGEX 3T (Amersham Pharmacia Biotech). GST and GST-fusion proteins were expressed in *E.coli* strain TOP10 essentially

as described (Frangioni & Neel, Anal. Biochem. 210 (1993), 179 - 187). For pull down experiments 10 cm dishes of confluent endothelial cells were pretreated with pervanadate, lysed and incubated with 10 µg of GST-fusion protein prebound to glutathion-sepharose as described before (Jallal et al., J. Biol. Chem. 272 (1997), 12158 - 12163).

Fig. 3b shows co-immunoprecipitation of VE-PTP trapping mutants (C->S, R->A) with Tie-2. COS-1 cells were transfected with FLAG-tagged VE-PTP and trapping mutants together with Tie-2. Immunoprecipitation was performed with anti-FLAG antibody M2. Precipitates were blotted with a Tie-2 specific monoclonal antibody.

pCMV-FLAG VE-PTP wt, C->S and R->A contain cDNA sequences coding for a polypeptide stretch corresponding to aa 1418-1977 in HPTPβ cloned in pCMV-FLAG-1 (Kodak). Trapping mutations C->S and R->A were introduced by PCR mutagenesis using primer Prbetamutcs 5'-TCC GTA GTG CAC TCG AGT GCT GGT GTG-3' and primer Prbetamutra 5'-GCT GGT GTG GGC GCC ACA GGG ACG TTC-3'. COS-1 cells (Gluzman, Cell 23 (1981), 175 - 182) were transfected using the modified calcium phosphate method (Chen & Okayama, Mol. Cell. Biol. 7 (1987), 2745 - 2752). For transfection 10 µg of pCMV-FLAG derivates and 2 µg of expression plasmids coding for the RTKs were used. As control 0.5 µg of EGFP expression plasmid (Clontech) were cotransfected. Cells were harvested after 2 d of expression. Transfection efficiency was evaluated under fluorescent light and was usually between 30 - 70%.

In mixing experiments of endothelial cell lysates and trapping mutants of the VE-PTP catalytic domain fused to GST, we detected interaction with the Tie-2 receptor, while GST alone did not precipitate Tie-2. The interaction was independent of pretreatment with pervanadate. In these assays coprecipitation f VEGFR-2 was never detectable (Fig. 3a).

To test for potential substrate interactions with Tie-2 and VEGFR-2 we coexpressed these RTKs with either a FLAG-tagged version of VE-PTP corresponding to aa 1418-1997 of HPTPβ, or the respective trapping mutants (Fig. 1a). Physical association was analysed by co-immunoprecipitation using an anti-FLAG-antibody and subsequent blotting of the precipitates with antibodies specific for the repective RTK. In this assay the Tie-2 receptor co-precipitated with both trapping mutants of VE-PTP (C- > S, R- > A) (Fig. 3b). The wild type phosphatase failed to precipitate Tie-2 efficiently, even though the receptor was expressed at comparable levels. This reduced association of PTPs *in vitro* with their substrates is due to the transient nature of the enzyme substrate association. Unlike Tie-2, VEGFR-2 could neither be co-immunoprecipitated with VE-PTP nor with one of the trapping mutants, even though VEGFR-2 expression was comparable to that of Tie-2.

Example 5

Finally, the phosphorylation state of RTKs was determined in the presence of VE-PTP. Figure 4 shows dephosphorylation of (a) Tie-2 but not (b) VEGR-2 by wild-type VE-PTP. RTKs were immunoprecipitated with specific antibodies from cotransfected COS-1 cells. Precipitates were blotted with anti-phosphotyrosine antibodies and after stripping reprobed with RTK-specific antibodies.

Tie-2 and VEGFR-2 expression vectors were published previously (Koblizek et al., Curr. Biol. 8 (1997), 529 - 532; Millauer et al., Cell 72 (1993), 835 - 846). Rat monoclonal antibodies against Tie-2 clones 3g1 and 4g8 (Koblizek et al. (1997) supra) and Flk-1 clone 12σ1 (Kataoka et al., Devel. Growth Diff. 39 (1997), 729 - 740) were used. Immunoprecipitations were performed with 5 μg of the monoclonal antibodies and immunoblotting with 2 μg/ml. Polyclonal anti-Flk-1 serum 1D3 (Sugen) was used in a 1:5000 dilution. Monoclonal anti-Flag antibody M2 (Kodak), polyclonal antiserum

against Tie-2 (Santa Cruz Bi technology) and monoclonal mouse antibody against phosphotyrosine PY20 (Transduction Labs) were used according to the manufacturer's instructions. Immunoprecipitations and immunoblotting were performed as described before (Esser et al., J. Cell. Biol. 140 (1998), 947 - 959); Jallal et al., J. Cell. Biol. Chem. 272 (1997), 12158 - 12162).

Immunoprecipitates of VEGFR-2 and Tie-2 co-expressed with either the VE-PTP trapping mutants (C->S, R->A) or wt VE-PTP were blotted with an α-phosphotyrosine-specific antibody and then reprobed with antibody specific for the RTK. Only for Tie-2, changes in the phosporylation status were observed. In the presence of the trapping mutants (C->S, R->A) the receptor was reproducibly more highly phosphorylated than in the controls. This hyperphosphorylation of Tie-2 in the presence of catalytically impaired trapping mutants suggests that physical interaction leads to protection of the receptor from dephosphorylation. In contrast, hypophosphorylation of the Tie-2 receptor was observed in the presence of wt VE-PTP, when compared to vector control (Fig. 4a). No significant changes were detected in the phosphorylation status of VEGFR-2, irrespective of the presence of VE-PTP or its trapping mutants (Fig. 4b). These findings clearly show that Tie-2 is a specific substrate for the endothelial specific phosphatase VE-PTP.

Claims

1. Use of vertebrate vascular-endothelial protein-tyrosine phosphatases or portions thereof for the manufacture of an agent for monitoring or modulating the activity of receptor-type tyrosine kinase Tie-2.

2. The use of claim 1 wherein said phosphatase is selected from murine phosphatase VE-PTP, human phosphatase HPTPβ or portions thereof.

3. The use of claim 1 or 2 wherein said portion comprises the catalytic domain.

4. Use of nucleic acids encoding vertebrate vascular-endothelial protein-tyrosine phosphatases or portions thereof for the manufacture of an agent for monitoring or modulating the activity of receptor-type tyrosine kinase Tie-2.

5. The use of claim 4 wherein said nucleic acid comprises at least 15 nucleotides from murine phosphatase VE-PTP nucleic acid, human phosphatase HPTPβ nucleic acid or sequences complementary thereto.

6. The use of ligands for vertebrate vascular-endothelial protein-tyrosine phosphatases for the manufacture of an agent for monitoring or modulating the activity of receptor-type tyrosine kinase Tie-2.

7. The use of claim 7 wherein said ligands are selected from antibodies and antibody fragments.

8. The use of any one of claims 1 - 7 for detecting Ti -2 activity.

9. The use f any one of claims 1 - 7 for stimulating Tie-2 activity.

10. The use of any one of claims 1 - 7 for repressing Tie-2 activity.

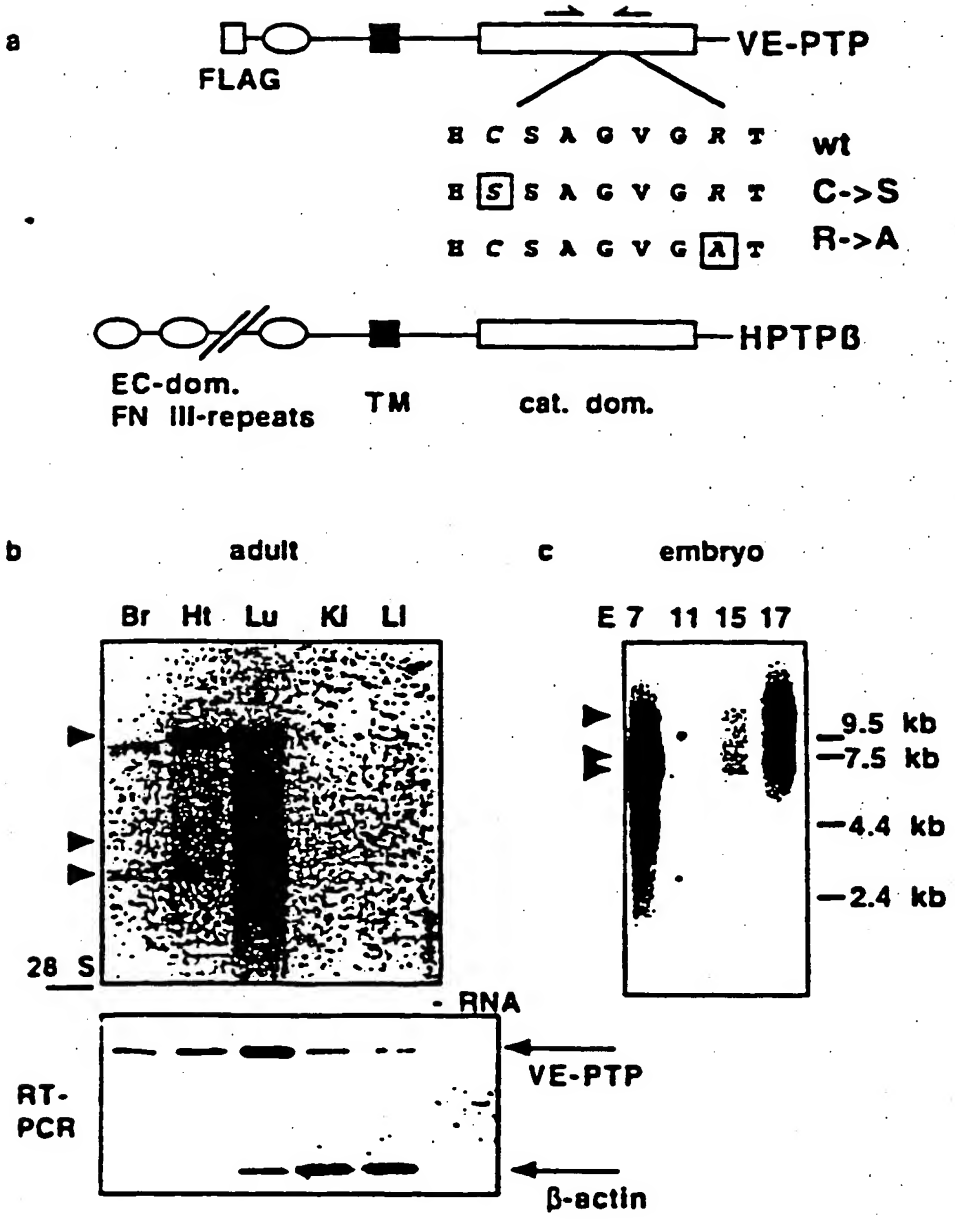
11. The use of any one of the previous claims for monitoring or modulating angiogenesis.

12. The use of any one of the previous claims for inducing vascular growth or remodelling and blood vessel maturation.

13. The use of any one of the previous claims for inhibiting vascular growth or remodelling and blood vessel maturation.

14. The use of any one of the previous claims for inhibiting tumor growth.

15. The use of any one of the previous claims for inhibiting formation of tumor metastases.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H S S A G V G R T C->S
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FN III-repeats
TM
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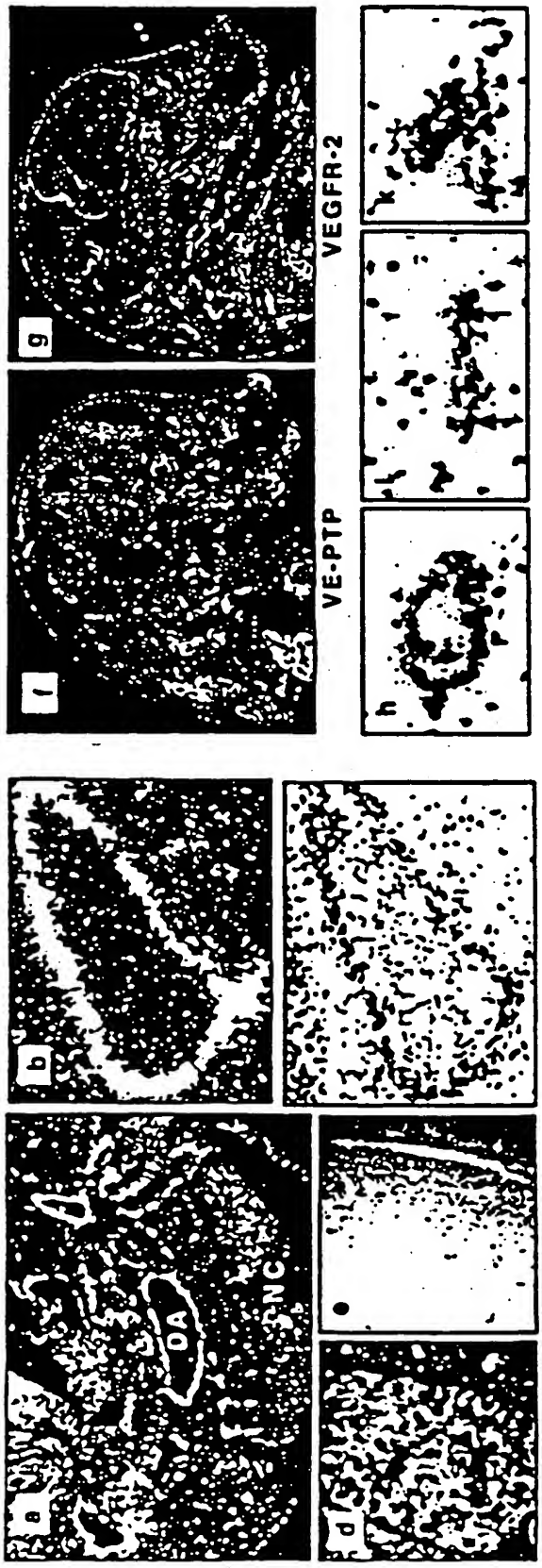
Fig. 1



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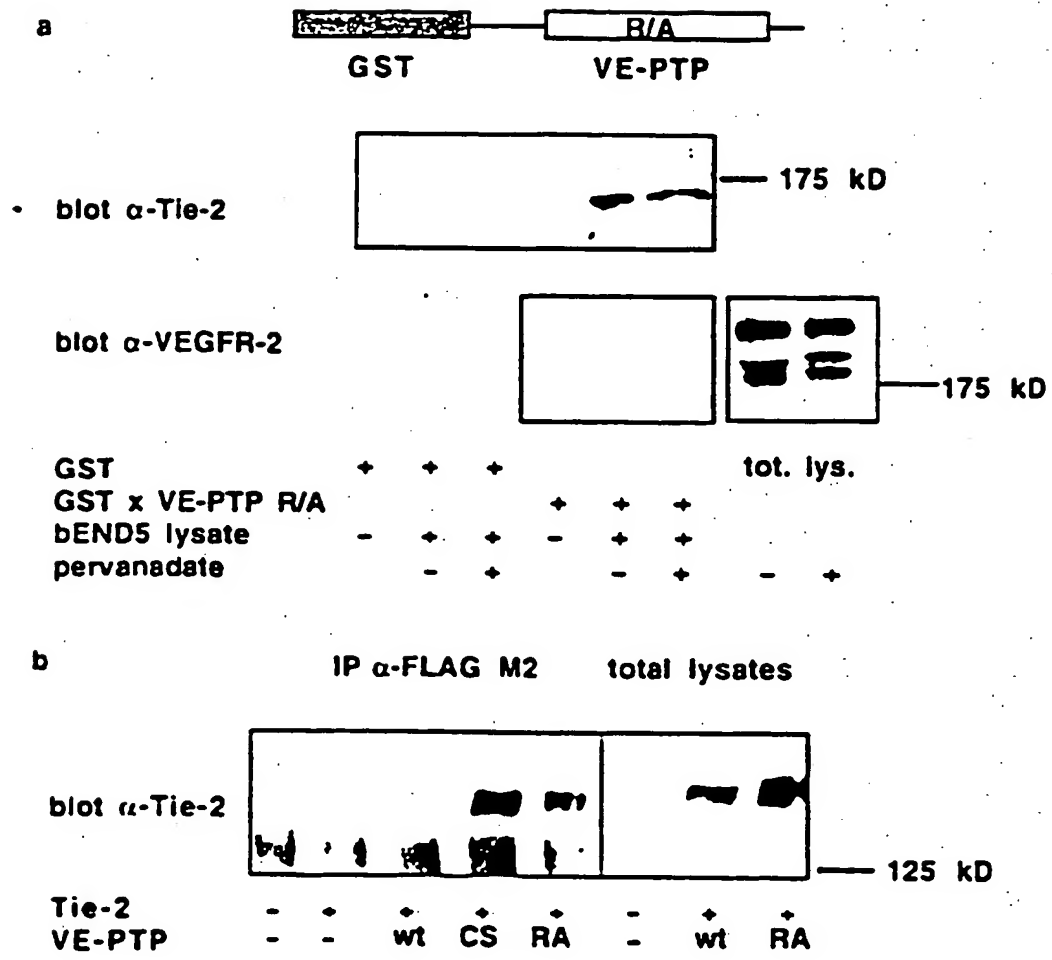
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pervanadate
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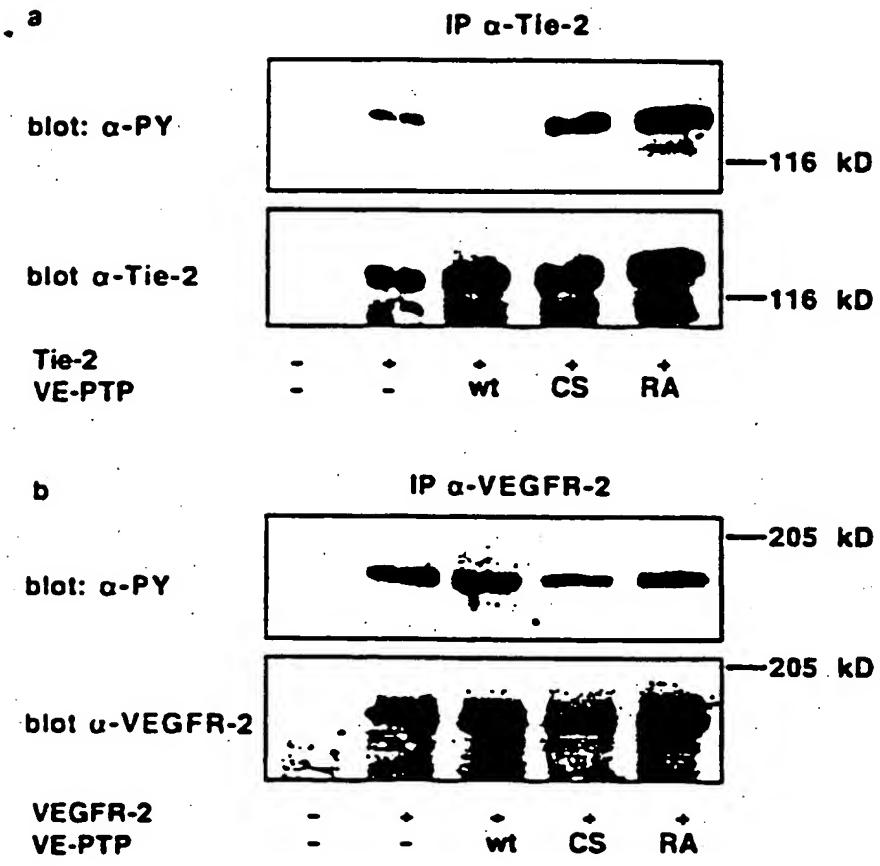
Fig. 3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VE-PTP
- + + + +
- - wt CS RA
b
IP α-VEGFR-2
blot: α-PY
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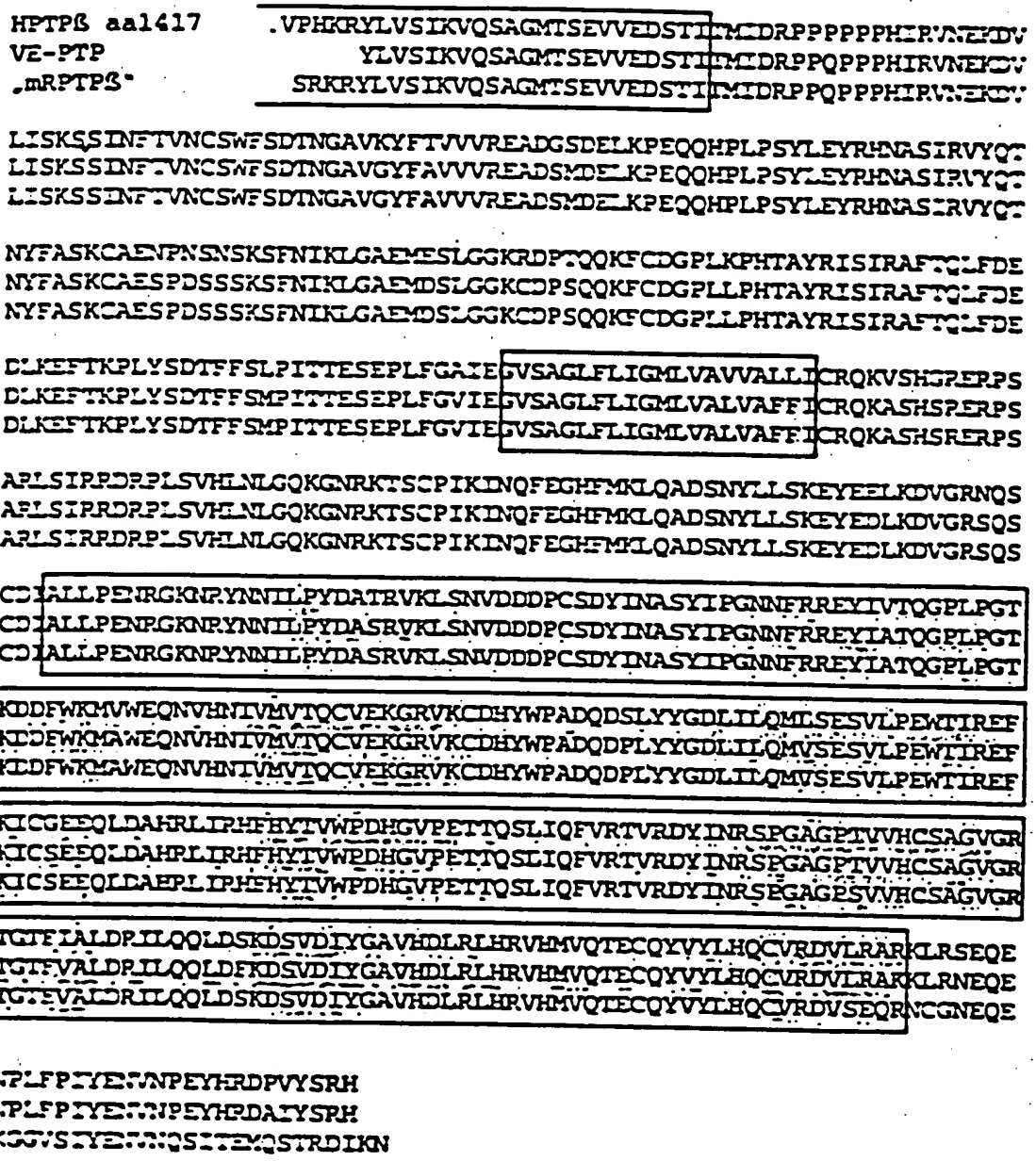
VEGFR-2
VE-PTP
- + + + +
- - wt CS RA

Fig. 4

Fig. 5



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Glu Val Val Glu Asp Ser Thr Ile Thr Met Ile Asp Arg Pro Pro Gln
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Pro Pro Pro His Ile Arg Val Asn Glu Lys Asp Val Leu Ile Ser Lys
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tct tcc atc aac ttt act gtc aac tgc agc tgg ttc agc gac acc aac  192
Ser Ser Ile Asn Phe Thr Val Asn Cys Ser Trp Phe Ser Asp Thr Asn
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Asp Glu Leu Lys Pro Glu Gln Gln His Pro Leu Pro Ser Tyr Leu Glu
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85 90 95

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agc aaa tgt gct gaa agt ccc gac agc agt tct aaa agt ttc aac att  384
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aag ctt gga gca gag atg gac agc ctc ggt ggc aaa tgt gat ccc agt  432
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Leu Phe Leu Ile Gly Met Leu Val Ala Leu Val Ala Phe Phe Ile Cys
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Val Trp Pro Asp His Gly Val Pro Glu Thr Thr Gln Ser Leu Ile Gln
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90 95 100

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Gly Lys Val Thr Ser Tyr Glu Val Gln Leu Phe Asp Glu Asn Asn Gln
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Lys Ile Gln Gly Val Gln Ile Gln Glu Ser Thr Ser Trp Asn Glu Tyr
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Arg Leu Met Leu Met Asp Lys Gly Ile Leu Val His Gly Gly Val Val
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Asp Lys His Ala Thr Ser Tyr Ala Phe His Gly Leu Ser Pro Gly Tyr
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Leu Tyr Asn Leu Thr Val Met Thr Glu Ala Ala Gly Leu Gln Asn Tyr
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Val Ser Gly Glu Leu Ser Ala Gln Lys Met Ala Val Gly Arg Thr Phe
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Pro Asp Lys Val Ala Asn Leu Glu Ala Asn Asn Asn Gly Arg Met Arg
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Ser Leu Val Val Ser Trp Ser Pro Pro Ala Gly Asp Trp Glu Gln Tyr
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Arg Ile Leu Leu Phe Asn Asp Ser Val Val Leu Leu Asn Ile Thr Val
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425                 430                 435                 440

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Gly Arg Gln Tyr Glu Val Glu Val Ile Val Glu Ser Gly Asn Leu Lys
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Thr Ile Pro Lys His Val Phe Glu His Thr Phe His Arg Leu Glu Ala
        955                 960                 965

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caa aaa gct gct ggt gtg gca gaa aga tat gat atc ctg ctt cta act   3126
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gaa aat gga atc ctt ctg cgc aac aca tca gag cca gcc act act aag   3174
Glu Asn Gly Ile Leu Leu Arg Asn Thr Ser Glu Pro Ala Thr Thr Lys
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caa cac aaa ttt gaa gat cta aca cca ggc aag aaa tac aag ata cag   3222
Gln His Lys Phe Glu Asp Leu Thr Pro Gly Lys Lys Tyr Lys Ile Gln
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atc cta act gtc agt gga ggc ctc ttt agt aag gaa gcc cag act gaa   3270
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Ile Leu Thr Val Ser Gly Gly Leu Phe Ser Lys Glu Ala Gln Thr Glu
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ggc cga aca gtc cca gca gct gtc acc gac ctg agg atc aca gag aac   3318
Gly Arg Thr Val Pro Ala Ala Val Thr Asp Leu Arg Ile Thr Glu Asn
                1085                1090                1095

tcc acc agg cac ctg tct ttc cgc tgg acc gcc tca gag ggg gag ctc   3366
Ser Thr Arg His Leu Ser Phe Arg Trp Thr Ala Ser Glu Gly Glu Leu
            1100                1105                1110

agc tgg tac aac atc ttt ttg tac aac cca gat ggg aat ctc cag gag   3414
Ser Trp Tyr Asn Ile Phe Leu Tyr Asn Pro Asp Gly Asn Leu Gln Glu
        1115                1120                1125

aga gct caa gtt gac cca cta gtc cag agc ttc tct ttc cag aac ttg   3462
Arg Ala Gln Val Asp Pro Leu Val Gln Ser Phe Ser Phe Gln Asn Leu
    1130                1135                1140

cta caa ggc aga atg tac aag atg gtg att gta act cac agt ggg gag   3510
Leu Gln Gly Arg Met Tyr Lys Met Val Ile Val Thr His Ser Gly Glu
1145                1150                1155                1160

ctg tct aat gag tct ttc ata ttt ggt aga aca gtc cca gcc tct gtg   3558
Leu Ser Asn Glu Ser Phe Ile Phe Gly Arg Thr Val Pro Ala Ser Val
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agt cat ctc agg ggg tcc aat cgg aac acg aca gac agc ctt tgg ttc   3606
Ser His Leu Arg Gly Ser Asn Arg Asn Thr Thr Asp Ser Leu Trp Phe
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aac tgg agt cca gcc tct ggg gac ttt gac ttt tat gag ctg att ctc   3654
Asn Trp Ser Pro Ala Ser Gly Asp Phe Asp Phe Tyr Glu Leu Ile Leu
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tat aat ccc aat ggc aca aag aag gaa aac tgg aaa gac aag gac ctg   3702
Tyr Asn Pro Asn Gly Thr Lys Lys Glu Asn Trp Lys Asp Lys Asp Leu
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acg gag tgg cgg ttt caa ggc ctt gtt cct gga agg aag tac gtg ctg   3750
Thr Glu Trp Arg Phe Gln Gly Leu Val Pro Gly Arg Lys Tyr Val Leu
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tgg gtg gta act cac agt gga gat ctc agc aat aaa gtc aca gcg gag   3798
Trp Val Val Thr His Ser Gly Asp Leu Ser Asn Lys Val Thr Ala Glu
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agc aga aca gct cca agt cct ccc agt ctt atg tca ttt gct gac att   3846
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Ser Arg Thr Ala Pro Ser Pro Pro Ser Leu Met Ser Phe Ala Asp Ile
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Ala Asn Thr Ser Leu Ala Ile Thr Trp Lys Gly Pro Pro Asp Trp Thr
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gac tac aac gac ttt gag ctg cag tgg ttg ccc aga gat gca ctt act   3942
Asp Tyr Asn Asp Phe Glu Leu Gln Trp Leu Pro Arg Asp Ala Leu Thr
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gtc ttc aac ccc tac aac aac aga aaa tca gaa gga cgc att gtg tat   3990
Val Phe Asn Pro Tyr Asn Asn Arg Lys Ser Glu Gly Arg Ile Val Tyr
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ggt ctt cgt cca ggg aga tcc tat caa ttc aac gtc aag act gtc agt   4038
Gly Leu Arg Pro Gly Arg Ser Tyr Gln Phe Asn Val Lys Thr Val Ser
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Gly Asp Ser Trp Lys Thr Tyr Ser Lys Pro Ile Phe Gly Ser Val Arg
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aca aag cct gac aag ata caa aac ctg cat tgc cgg cct cag aac tcc   4134
Thr Lys Pro Asp Lys Ile Gln Asn Leu His Cys Arg Pro Gln Asn Ser
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acg gcc att gcc tgt tct tgg atc cct cct gat tct gac ttt gat ggt   4182
Thr Ala Ile Ala Cys Ser Trp Ile Pro Pro Asp Ser Asp Phe Asp Gly
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Tyr Ser Ile Glu Cys Arg Lys Met Asp Thr Gln Glu Val Glu Phe Ser
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aga aag ctg gag aaa gaa aaa tct ctg ctc aac atc atg atg cta gtg   4278
Arg Lys Leu Glu Lys Glu Lys Ser Leu Leu Asn Ile Met Met Leu Val
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ccc cat aag agg tac ctg gtg tct atc aaa gtg cag tcg gcc ggc atg   4326
Pro His Lys Arg Tyr Leu Val Ser Ile Lys Val Gln Ser Ala Gly Met
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act agc gag gtg gtt gaa gac agc act atc aca atg ata gac cgc ccc   4374
Thr Ser Glu Val Val Glu Asp Ser Thr Ile Thr Met Ile Asp Arg Pro
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Ser Lys Ser Ser Ile Asn Phe Thr Val Asn Cys Ser Trp Phe Ser Asp
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acc aat gga gct ctg aaa tac ttc aca gtg ctg ctg aga gag gct gat   4518
Thr Asn Gly Ala Val Lys Tyr Phe Thr Val Val Val Arg Glu Ala Asp
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ggc agt gat gag ctg aag cca gaa cag cag cac cct ctc cct tcc tac   4566
Gly Ser Asp Glu Leu Lys Pro Glu Gln Gln His Pro Leu Pro Ser Tyr
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Leu Glu Tyr Arg His Asn Ala Ser Ile Arg Val Tyr Gln Thr Asn Tyr
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Phe Ala Ser Lys Cys Ala Glu Asn Pro Asn Ser Asn Ser Lys Ser Phe
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aac att aag ctt gga gca gag atg gag agc tta ggt gga aaa cgc gat   4710
Asn Ile Lys Leu Gly Ala Glu Met Glu Ser Leu Gly Gly Lys Arg Asp
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ccc act cag caa aaa ttc tgt gat gga cca ctg aag cca cac act gcc   4758
Pro Thr Gln Gln Lys Phe Cys Asp Gly Pro Leu Lys Pro His Thr Ala
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Tyr Arg Ile Ser Ile Arg Ala Phe Thr Gln Leu Phe Asp Glu Asp Leu
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aag gaa ttc aca aag cca ctc tat tca gac aca ttt ttt tct tta ccc   4854
Lys Glu Phe Thr Lys Pro Leu Tyr Ser Asp Thr Phe Phe Ser Leu Pro
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atc act att gaa tca gag ccc ttg ttt gga gct att gaa ggt gtg agt   4902
Ile Thr Thr Glu Ser Glu Pro Leu Phe Gly Ala Ile Glu Gly Val Ser
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gct ggt ctg ttt tta att ggc atg cta gtg gct gtt gtt gcc tta ttg   4950
Ala Gly Leu Phe Leu Ile Gly Met Leu Val Ala Val Val Ala Leu Leu
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Leu Ser Ile Arg Arg Asp Arg Pro Leu Ser Val His Leu Asn Leu Gly
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cag aaa ggt aac cgg aaa act tct tgt cca ata aaa ata aat cag ttt   5094
Gln Lys Gly Asn Arg Lys Thr Ser Cys Pro Ile Lys Ile Asn Gln Phe
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Glu Gly His Phe Met Lys Leu Gln Ala Asp Ser Asn Tyr Leu Leu Ser
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aag gaa tac gag gag tta aaa gac gtg ggc cga aac cag tca tgt gac   5190
Lys Glu Tyr Glu Glu Leu Lys Asp Val Gly Arg Asn Gln Ser Cys Asp
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Ile Ala Leu Leu Pro Glu Asn Arg Gly Lys Asn Arg Tyr Asn Asn Ile
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ttg ccc tat gat gcc acg cga gtg aag ctc tcc aat gta gat gat gat   5286
Leu Pro Tyr Asp Ala Thr Arg Val Lys Leu Ser Asn Val Asp Asp Asp
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Pro Cys Ser Asp Tyr Ile Asn Ala Ser Tyr Ile Pro Gly Asn Asn Phe
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Val Thr Gln Cys Val Glu Lys Gly Arg Val Lys Cys Asp His Tyr Trp
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Cys Gly Glu Glu Gln Leu Asp Ala His Arg Leu Ile Arg His Phe His
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tat acg gtg tgg cca gac cat gga gtc cca gaa acc act cag tct ctg   5670
Tyr Thr Val Trp Pro Asp His Gly Val Pro Glu Thr Thr Gln Ser Leu
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atc cag ttt gtg aga act gtc agg gac tac atc aac aga agc ccg ggt   5718
Ile Gln Phe Val Arg Thr Val Arg Asp Tyr Ile Asn Arg Ser Pro Gly
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Ala Gly Pro Thr Val Val His Cys Ser Ala Gly Val Gly Arg Thr Gly
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acc ttt att gca ttg gac cga atc ctc cag cag tta gac tcc aaa gac   5814
Thr Phe Ile Ala Leu Asp Arg Ile Leu Gln Gln Leu Asp Ser Lys Asp
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Ser Val Asp Ile Tyr Gly Ala Val His Asp Leu Arg Leu His Arg Val
    1930                1935                1940

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1945                1950                1955                1960

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Arg Asp Val Leu Arg Ala Arg Lys Leu Arg Ser Glu Gln Glu Asn Pro
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INTERNATIONAL SEARCH REPORT

Inter ...nal Application No
PCT/EP 00/03613

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/42 A61K38/46 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, EPO-Internal, CHEM ABS Data, BIOSIS, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HUANG L ET AL: "GRB2 and SH-PTP2: potentially important endothelial signaling molecules downstream of the TEK/ TIE2 receptor tyrosine kinase." ONCOGENE, (1995 NOV 16) 11 (10) 2097-103., XP002117444 the whole document	1,4,6
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[X] * other documents are listed in the continuation of box C

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Date of the actual completion of the international search	Date of mailing of the international search report
8 September 2000	19/09/2000

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
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